



Precoating of alloplastic materials with living human fibroblasts

A feasibility study

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Abstract

Background: The use of alloplastic materials such as polypropylene and polyester has reduced the recurrence of abdominal wall hernias. Concomitantly, new problems have arisen such as inflammatory response against the implanted material and the development of enteric fistulas in case of direct contact of the bowel to polypropylene and polyester. A precoating of the PP with collagen and other absorbable materials seems to reduce the incidence of adhesions and fistulas. The aim of this study was to show the technical feasibility of a precoating of polypropylene with living human fibroblasts and to investigate the growth properties of the cells under these conditions in vitro.

Methods: The textile structure of three different alloplastic materials is described (SurgiPro[®], TycoHealthcare; Parietene3 PP1510[®], Dallhausen; VIPRO II[®], Ethicon Endosurgery). Enhanced Green Fluorescence Protein (EGFP) transduced human foreskin fibroblasts (KiF5) were seeded onto these different alloplastic materials. Proliferation was analyzed by FACS analysis of Ki67 expression. The coating process of the whole mesh area was observed over time with UV-light microscopy, immunostaining, and scanning electron microscopy (SEM). The expression of collagen type I and III was investigated by immunostaining.

Results: The three alloplastic materials used were knitted fabrics with different textile structures. KiF5 colonized the entire alloplastic material within 4–6 weeks. Cells were proliferating, as detected by Ki67 expression. SEM showed surface ruffles and long cellular extensions, indicating an active cell metabolism. Light microscopy and SEM suggested that the cells modify the apolar surface by deposition of extracellular matrix components before colonization.

Conclusion: Our study shows the feasibility of precoating of polypropylene meshes with living human fibro-

blasts and opens the possibility for clinical use in the future.

Key words: Alloplastic materials — Scaffolds — Fibroblasts — Precoating — Hernia repair

The most important quality parameter in hernia surgery is the recurrence rate, which was a major problem in the prealloplastic era. The mean reason for recurrence is tension during scarring and the process of abdominal wall stabilization. A sufficiently tension-free surgical technique with the opportunity of bridging even great abdominal wall defects became possible for the first time with the introduction of alloplastic materials. The use of alloplastic materials such as polypropylene (PP) and polyester (PE) has revolutionized surgical hernia therapy during recent decades. Along with the benefit of a reduced recurrence rate with the use of alloplastic materials, new problems arose [16]. Although increased scarring was prevented by use of so-called low weight meshes, the problem of local inflammatory reaction and the development of seroma persisted. Furthermore, the reason for the surface destruction described for removed meshes remains unclear [8]. Laparoscopic repair of abdominal wall hernias might be superior to the conventional technique [7, 19] but the development of adhesions and intestinal fistulas in case of direct contact to PP led to a drawback for this elegant technique [14, 24]. Alternatively, use of expanded polytetrafluoroethylene (ePTFE) has been recommended, which did not present a solution for all problems, especially concerning the high prices for the materials used. Coating with biodegradable materials or with cell proliferation-inducing substances seemed to improve the incorporation of alloplastic material [1, 11]. An extracorporeal precoating of PP with living human fibroblasts has not been investigated so far. The aim of this study was to investigate the technical feasibility of growing fibro-

blasts on PP meshes and to introduce a model system for examinations of fibroblasts directly on PP.

Materials and methods

Structure analysis

The textile structure of three PP meshes were investigated: SurgiPro[®] (Tyco Healthcare), Parietene3 PP1510[®] (Dallhausen), and VIPRO II[®] (Ethicon Endosurgery, all Germany). The meshes were analyzed using a stereo light microscope (SZH, Olympus, Germany).

Cell culture

Enhanced green fluorescence protein (EGFP) transduced human foreskin fibroblasts (KiF5) were described previously [17]. Cells were cultured in tissue culture flasks (Nunc, Germany) in RPMI-1640 medium supplemented with 10% fetal calf serum (PAA, Germany), 2 mM glutamine, 1mM sodium pyruvate (Gibco, Germany) at 37°C in a humid atmosphere under 5% carbon dioxide (CO₂). Cells were washed twice with PBS, detached from the culture flask with trypsin, and counted in an automatic cell counter CASY1 (Schaerfe System, Germany). Then, 300,000 cells/ml were seeded in drop form on small pieces (0.5 × 0.5 cm) of the various alloplastic materials placed in six-well plates with a low amount of culture medium to allow adherence. After 12 h fresh culture medium (2 ml) was added. Cell growth was observed with a microscope equipped for fluorescence microscopy to visualize EGFP.

Fluorescence activated cell sorting (FACS) analysis

For FACS analyses the cells were cultivated on alloplastic material for 8 days. Cells as monolayer were cultivated for 4 days. Cells were harvested from the mesh and from the monolayer by treatment with 0.25% trypsin/0.02% EDTA in PBS (Gibco, Germany) for 5 min at 37°C. The removal of the cells was observed immediately by light microscope. Cells were then washed and resuspended in PBS for further processing. To detect the expression of Ki67 a procedure was used as described previously [10]. Briefly, aliquots of the cells suspension (500 μ l at a concentration of 500,000 cells) were fixed by adding 50 μ l of a 2% solution of paraformaldehyde in PBS for 10min on ice; cells were centrifuged (600g for 5 min) and resuspended in 500 μ l ice-cold PBS containing 0.1% Triton X-100, and incubated for 5 min on ice. Cells were resuspended in 100 μ l of PBS containing the MIB1 antibody (1:100, Dianova, Germany) or the IgG1 isotype control antibody (1:50, DAKO, Germany) for 45 min at 4°C. Cells were then washed by adding 1 ml of PBS, centrifuged, and resuspended in 100 μ l PBS containing FITC-conjugated rabbit anti-mouse immunoglobulin antiserum (1:50, Dako, Germany). After incubation for 45 min, cells were washed and resuspended in 1 ml PBS. The analysis was carried out using a FACScan and analyzed with the cell quest program (both Becton Dickinson Biosciences, Germany) [10]. At least 10,000 cells were examined for each determination.

Immunostaining

KiF5 cells were seeded on PP mesh pieces as described earlier, and allowed to grow for 2 weeks. Thereafter, the mesh pieces with adhering cells were washed twice with PBS and then fixed with acetone for 15 min. For blocking of endogenous peroxidase, incubation with 0.03% hydrogen peroxide (DAKO, Germany) was carried out for 20 min. Serum blocking and detection were performed with a peroxidase-based mouse staining kit (Vector Laboratories, USA). Nuclei were stained with hematoxyline. Monoclonal antibodies against human collagen type I and III were obtained from Loxo (Germany). The staining procedure followed the recommendation of the manufacturer. For negative control the primary antibody was omitted.

Scanning Electron Microscopy (SEM)

For SEM of cells cultivated on pieces of mesh for 1 week, an LEO 442 microscope was used (LEO Corp., Germany). The material was fixed with a modified Karnovsky solution overnight [18], then washed twice for 10 min with Soerensen buffer and subsequently incubated with a 1% aqueous osmium solution for 12 min. After dehydration in graded ethanol concentrations (50% to 100%, 2 × 20 min each), the material was dried to the critical point with liquid carbon oxide and coated with gold.

Results

Definition of the mesh structure

The different materials used in this study were investigated by light microscopy to reveal the mesh structure. SurgiPro showed a multifilament thread; the texture is pure filet weave. Differing dimensions of neighboring pores appear to be due to the absence of side connections between the filaments. This leads to pores with different forms. Parietene revealed a monofilament structure produced on a warp knitter machine with two guide bars creating a tricot texture. The meshes, lying obliquely, leads to a more diagonal stability of the mesh. VIPRO II represented a multifilament mesh produced by a warp knitter machine. The structure is a so-called honeycomb net. Inserted absorbable polyglactin filament does not participate in mesh formation but it improves the handling properties of the mesh during implantation.

Cell culture on the mesh

Light microscopy analysis showed cell growth between the filaments of the meshes. Normal transmission light microscopy, as well as detection of green fluorescence, shows cells adhering to the mesh structure. Figure 1 shows the mesh structure of Parietene (A–C) and SurgiPro (D–F) by normal light transmission with cells crossing the open pores after 1 week of culture (A and D). Investigation by fluorescence microscopy showing the green fluorescence of transduced EGFP reveals adherence of cells to the mesh structure after 2 weeks of culture (Fig. 1B, E) and the gradual filling the pores after 3 weeks of culture (Fig. 1C, F). Similar results were found for VIPRO II (data not shown). These data suggest active proliferation of KiF5 fibroblasts on PP meshes. An enlarged view of cells growing on SurgiPro mesh is shown in Fig. 1G as seen by light microscopy, and the corresponding EGFP fluorescence is shown in Fig. 1H, depicting the spread-out form of KiF5 fibroblasts on the mesh structure. Investigation using SEM in Fig. 1I revealed the spreading of cells on the PP mesh.

To test for proliferation, FACS analysis for expression of Ki67, a proliferation marker protein was performed. Figure 2 shows scan profiles for KiF5 cells from routine culture compared to KiF5 cells grown on the different mesh materials. Isotype controls are depicted in grey, KiF5 immunoreactivity is visualised by black

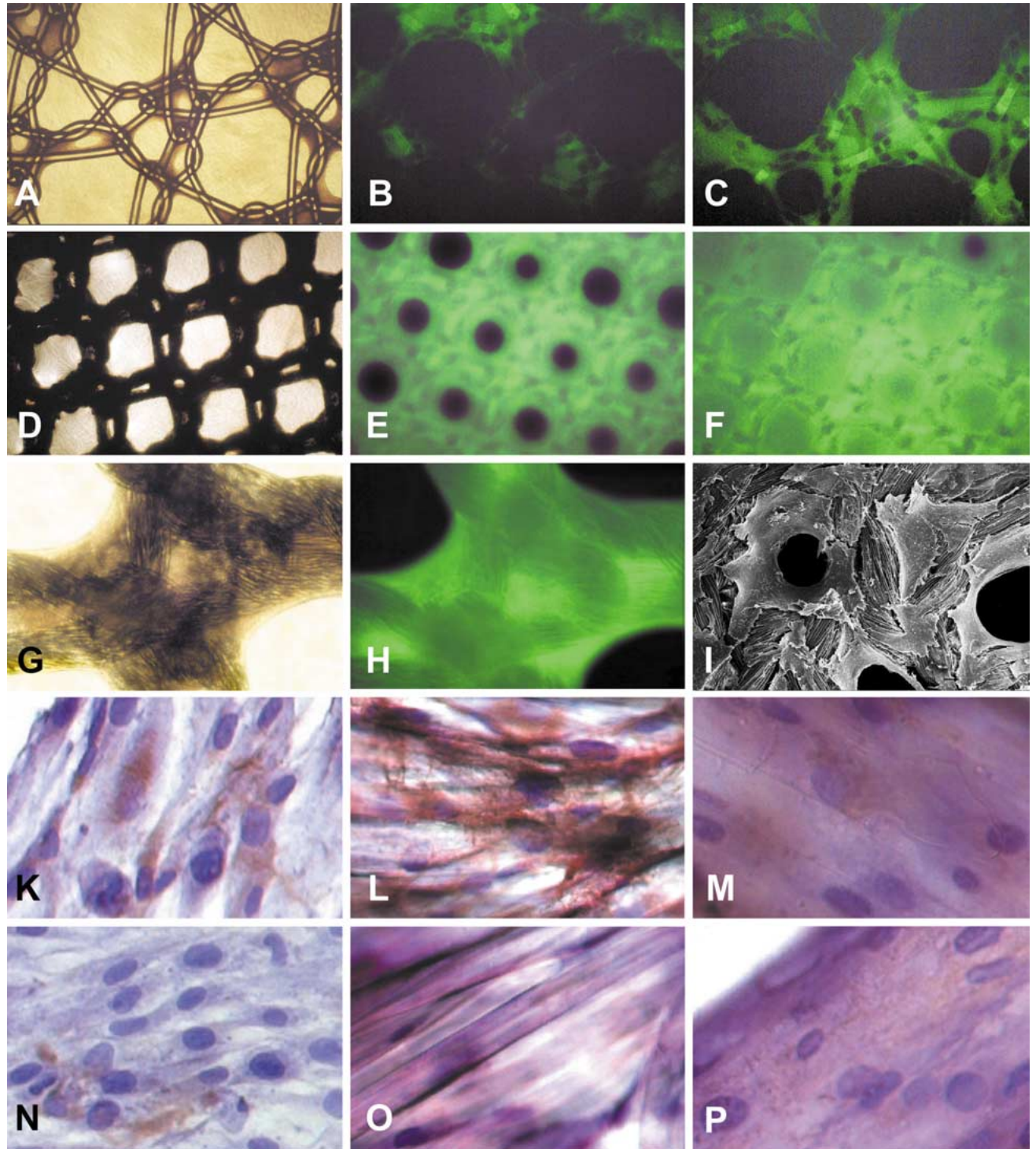


Fig. 1. Morphological characterisation of human fibroblasts grown on PP meshes (A–I, L, M, O, P) or as monolayer culture (K, N). KiF5 cultivated on Paritene (A–C) and SurgiPro (D–F) (A/D) transmission light microscopy after 1 week of culture (B/E) green fluorescence of KiF5 fibroblasts expressing EGFP after 2 weeks of culture. (C/F) green fluorescence of KiF5 fibroblasts expressing EGFP after 3 weeks of culture (all: original magnification 40×) (G–I) enlarged view of cells

growing on SurgiPro after 1 week (G) Transmission light microscopy. (H) Green fluorescence of KiF5 fibroblasts expressing EGFP (all: original magnification 100×) and (I) Scanning electron/Microscopy of KiF5 on SurgiPro. (K–P) Immunostaining against collagen type I (K–M) and collagen type III (N–P) (all magnification 200×) (K/N) KiF5 grown as monolayer cells. (L/O) KiF5 grown on SurgiPro (M/P) KiF5 grown on Paritene.

colouring of the curve. Standard culture grown fibroblast show a clear shift of Ki67-positive cells to the right. In comparison, KiF5 cells grown on PP meshes show a slightly less pronounced shift but are clearly positive for

Ki67 proliferation marker. Obviously, PP mesh-grown cells are more heterogeneous than cells from standard culture, as depicted by the slight distortion of the Gaussian distribution of the antibody-control cells.

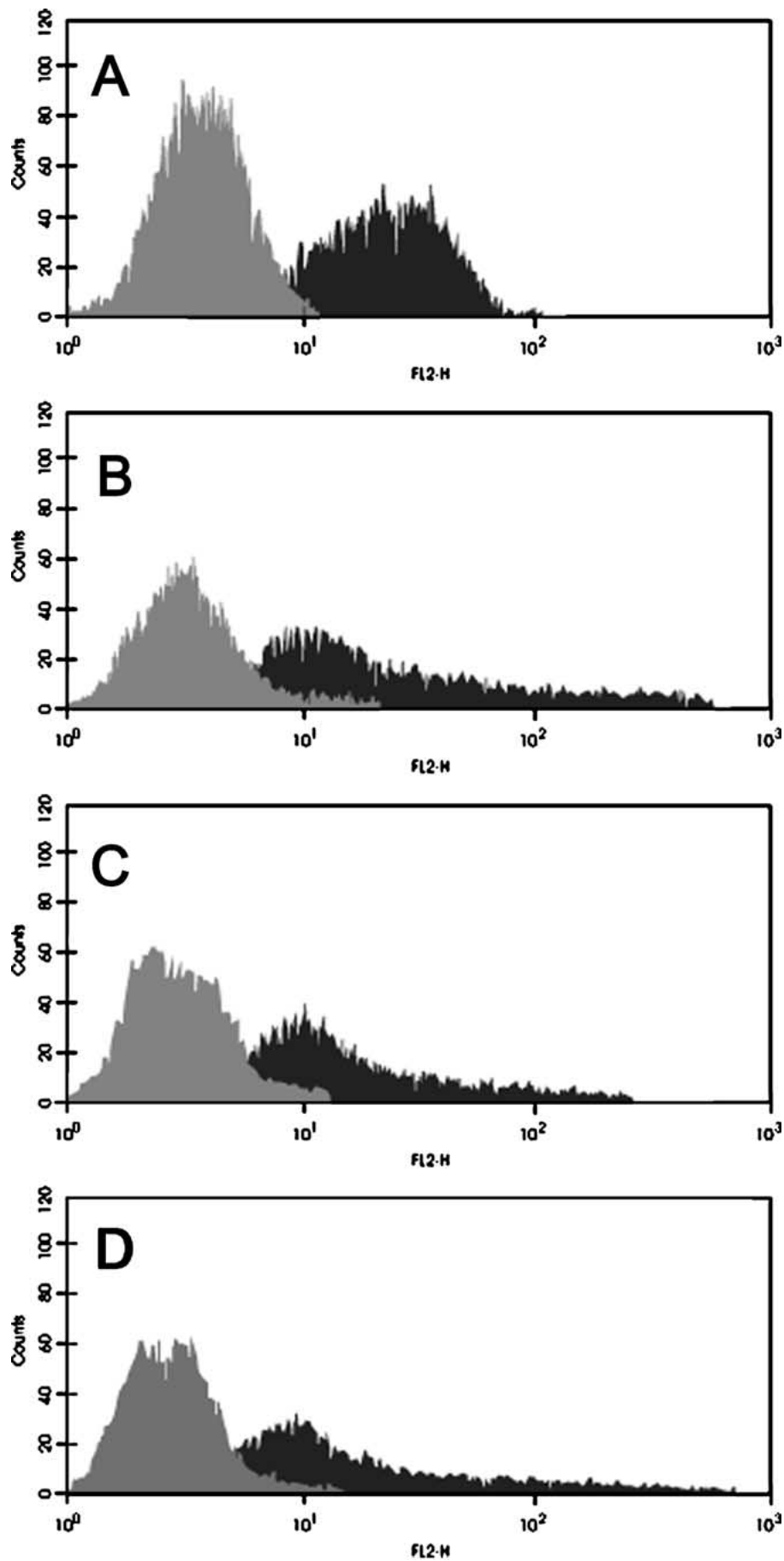


Fig. 2. FACS analysis of Ki67 expression in KiF5 fibroblasts: (A:) KiF5 after 4 days of monolayer culture. (B) KiF5 grown for 10 days on SurgiPro. (C) KiF5 grown for 10 days on Parietene (D) KiF5 grown for 10 days on VIPRO II. (Gray) isotype control (Black) Ki67 staining.

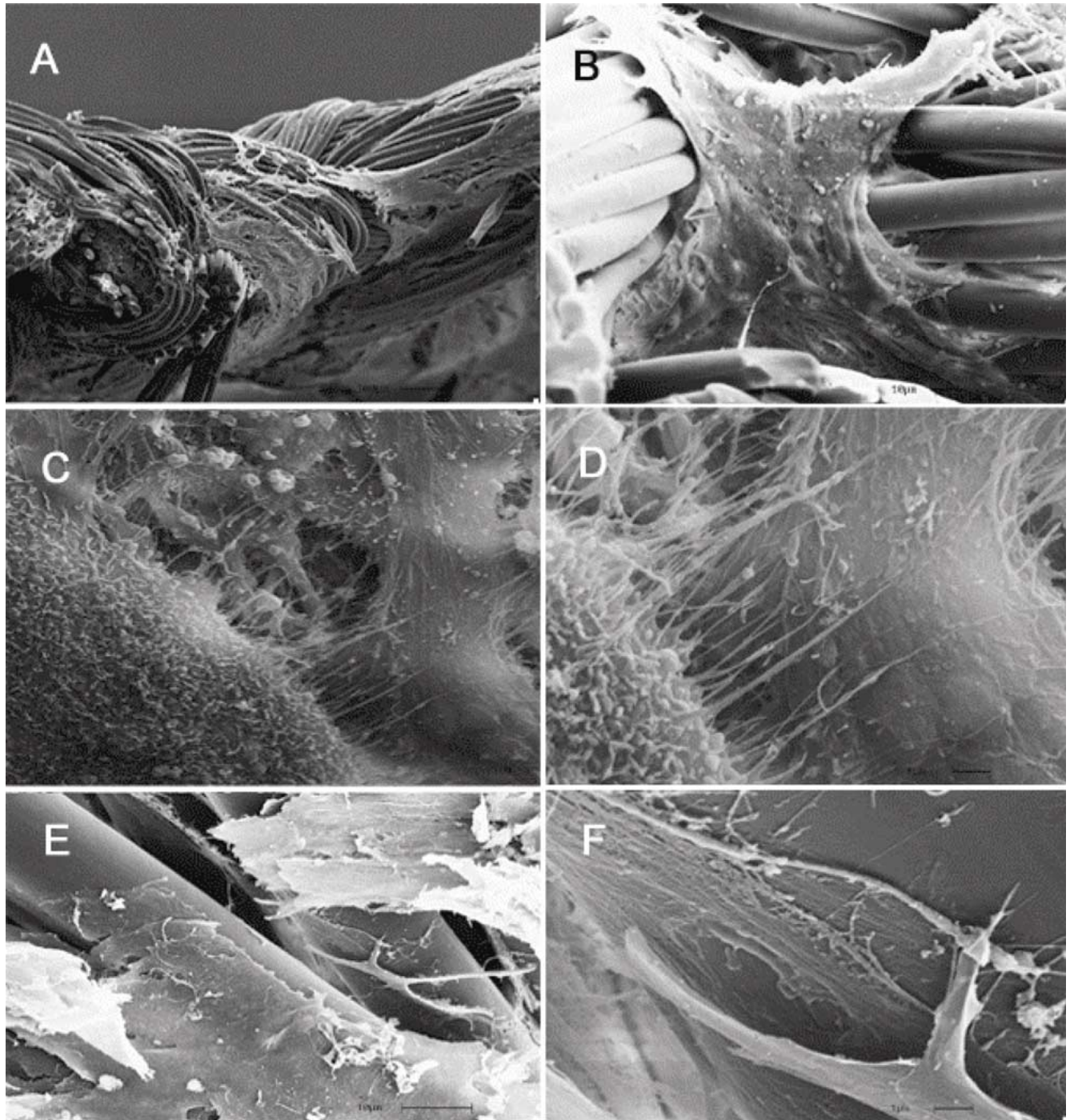


Fig. 3. SEM analysis of KiF5 fibroblasts growing on SurgiPro mesh. (A/B) Fibroblasts stretching over the mesh surface with extensions between individual fibres. (C/D) Fibroblasts showing numerous long cell extensions and surface ruffles (E/F) Deposition of ECM on PP by adherent fibroblasts (all SurgiPro).

SEM analysis confirmed that fibroblast cells settle on the PP surface covering separate PP-threads (Fig. 3A,B). Fibroblasts develop long extensions and show a ruffled surface, indicating viability, as dead cells would round up and detach (Fig. 3C,D). Furthermore, fibroblasts growing on PP modified the surrounding mesh surface by deposition of extracellular matrix (Fig. 3E,F).

To test for extracellular matrix compounds deposited by KiF5 fibroblasts, immunostaining for collagen

type I and III was carried out. Figure 1K–M shows staining of collagen I in monolayer-grown cells (Fig. 1K), and cells grown on SurgiPro (Fig. 1L) and Parietene mesh (Fig. 1M). Collagen III immunostaining is seen in Fig. 1N–P. Again monolayer cells were stained (Fig. 1O) and mesh-grown cells on SurgiPro (Fig. 1P) and Parietene (Fig. 1Q). Strongest reactivity was found for collagen type I, while collagen type III expression appears to be sporadic in monolayer cells (Fig. 1O)

and sparse in mesh grown specimen (Fig. 1P, Q). Collagen type 1 staining is found resembling an extended network of collagen fibres in cells grown on SurgiPro meshes (Fig. 1L).

Discussion

The physical chemical properties of PP (low density and high tenacity, high abrasion resistance, and resistance to acids and alkali) give them superiority compared to other alloplastic materials (PE or ePTFE). The thermofixation of the knitwear allows a high two-dimensional stability of the material. The fixed plane form is essential for the use as medical implant. The textile structure influences the form stability of the textile product. External pressure and distortion can influence the final form of the implanted material secondarily. Obviously, this is the reason for the so-called shrinkage, a reduced stabilized area after implantation of the alloplastic material. The development of implants is always a compromise between plastic and elastic properties. More flexibility improves the incorporation and prevents increased scarring plasticity, but includes a higher risk for secondary size reduction of the stabilized area, as well as the risk of hernia recurrence, and it worsens handling properties. The optimal mesh structure is not present in this time. All available meshes intend to combine form stability with flexibility.

Two major problems concerning the use of PP for hernia repair are under debate. First: the inflammatory response of the host against the implant [20]. Reasons and risk factors for different individual responses remain unclear. Development of so-called low-weight meshes seemed to reduce the inflammatory reaction of the organism against the implant. The results regarding advances of monofilament and multifilament meshes are conflicting [20]. A coating of the polypropylene surface with titanium seems to reduce the inflammatory response of the host against the alloplastic material. The reason for these different reactions remains unclear, too [20]. The second problem seems to be caused by the first: the development of adhesions and consecutive fistulas in case of direct contact between PP and bowel. Because of this observation the implantation of PP in the abdominal cavity during abdominal hernia repair is not recommended generally. A cost-intensive alternative is the use of ePTFE [3, 21, 26]. Another possibility is the precoating of PP with extracellular matrix (ECM) components such as collagen. These meshes are available as Sepramesh (Genzyme, Germany) [28]. Precoating with ECM appeared to reduce the incidence of adhesions but increases the infection rate [6, 24, 25]. The increased infection rate may be caused by the loss of hydrophobic surface of the PP, which prevents cell growth, but also bacterial infestation [27]. The difficulty for fibroblasts to grow directly on PP appears to be a major problem, because during the incorporation process the PP must be covered with ECM substances. After a period of embedding in ECM no new adhesions were observed [2]. The higher infection risk and the easier production process led to the development of

composite meshes consisting of PP for the stabilization of the abdominal wall and an absorbable layer directed to the abdominal cavity to prevent adhesions during the initial time of incorporation [24, 25]. One example is Proceed (Ethicon Endosurgery, Germany).

Recent *in vitro* investigations showed that adhesion-free areas in PP implants were carpeted with mesenchymal cells from the 5th day on. This appeared to prevent adhesions to PP and reduced inflammatory response [11]. These observations led us to investigate the growth of human fibroblasts on PP meshes *in vitro*. A precoating of PP meshes with fibroblasts could shorten the incorporation process because the fibroblasts have prepared the PP surface *in vitro*. Some *in vitro* model systems were described that are investigating the influence of PP on fibroblast growth, but in none of these systems were the cells seeded on the PP surface directly [5, 9]. Only an absorbable polyglycolacid mesh was used for precultivation with fibroblasts [13].

In the system presented here the cells proliferate directly on the material to be implanted and the fibroblasts colonized the entire alloplastic material. Analysis of Ki67 antigen expression, as a commonly used proliferation marker [10], showed some differences of cells grown on PP meshes compared to cells cultivated as monolayers, which was most likely due to the different growth conditions. Polystyrene, the material of cell culture dishes, is treated for better adherence of cells, whereas PP has no special cultivation properties. The cell population on PP was more inhomogeneous regarding the pattern of Ki67 expression, but reactivity for Ki67 in general indicated proliferation. In the study presented here, no differences in cellular growth were observed among the three different meshes. Viability of cells growing on PP meshes was supported by SEM analysis, which showed cells with numerous extensions and surface ruffles indicative of active cell metabolism. Apparently, production of extra cellular matrix components such as collagen is necessary for modification of the hydrophobic PP surface as the basis for cell colonisation. Immunostaining investigation showed a high expression of collagen type I and a minor expression of collagen type III. The collagen I to III ratio was shown in a rat model to be a marker for mesh incorporation and wound healing [12]. Type I collagen appears to be one of the major components for coating the PP. This agrees with other observations regarding matrix reorganization by fibroblasts *in vitro* [23]. For PP in a rat model, it was shown that an early coverage of the alloplastic material confers adhesion resistance [11]. One conclusion from these observations could be that implantation of precoated PP could prevent inflammation and fistula and lead to an earlier incorporation. The use of an experimental model system provides the means of investigating the reaction of cells toward different meshes. Furthermore, surface alterations of alloplastic materials during direct contact with living cells could be investigated. Within a period of 4–6 weeks no changes in surface quality like porous alterations or chipping of PP were observed in our system.

Human fibroblasts can be isolated and propagated *in vitro*. Tissue culture extension of autologous material is

already good clinical practice for keratinocytes in a therapeutic approach used for patients suffering from chronic leg ulcers [4, 15, 22]. This aspect opens the opportunity for a tailored approach for the patient with hernia repair in critical cases or large abdominal-wall defects. Fibroblasts from individual patients could be isolated and used for precoating of mesh material in vitro prior to implantation into the abdominal wall. To the best of our knowledge we have described for the first time the technical feasibility of coating a PP mesh with human fibroblasts. Further investigations are necessary for a better understanding of the inflammatory process and to show a reduced adhesion in vivo, employing an animal model.

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